

The Effect of Adding Organic Polymers on the Handling Properties, Strengths and Bioactivity of a Ca-Sr-Zn-Si Glass Polyalkenoate Cement

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ABSTRACT

This work demonstrates the addition of a number of naturally occurring proteins/polymers to a Zinc based Glass Polyalkenoate Cements (GPCs). Chitin (*Chi.*) Collagen (*Col.*), Cysteine (*Cys.*) and Keratin (*Ker.*) were added with the intention of improving the bioactivity of this cement. Initial testing involved characterization of the glass with X-ray Diffraction (XRD) and Differential Thermal Analysis (DTA) before and after sterilization with γ -irradiation. No significant changes occurred as a result of sterilization. Handling properties of the modified cements were not significantly different from those of the control, BT 101 (Working T_w - 36s, and Setting time T_s - 70s) except for *Chi.* (30s, $p \leq 0.016$) and *Cys.* (105s, $p \leq 0.0001$) respectfully. Comparison of the mechanical properties of BT 101 (compression - σ_c and biaxial flexural - σ_f) to the modified cements revealed a significant decrease in σ_c with *Chi.* and *Col.*, after 1, 7 and 30 days. However, there were little changes occurring in σ_f . Cement structural testing was investigated and found that the addition of these polymers greatly reduced the cements surface area, however the only significant change to occur in the solubility testing was *Ker.* ($p \leq 0.009$). Simulated Body Fluid (SBF) testing resulted in increased calcium phosphate (CaP) deposition of *Chi.* and *Col.* compared to BT 101. Cell culture studies determined only *Col.* significantly increased ($p \leq 0.0001$) in comparison to the control cement.

Keywords Glass Polyalkenoate Cement, Chitin, Collagen, Cysteine, Keratin.

1. INTRODUCTION

Glass Polyalkenoate Cements (GPCs) are traditionally used in dental applications as restorative and luting cements. However, they have potential as bone cements due to a number of attractive properties. They are reported to have excellent biocompatibility^[1], can adhere to surgical metals and the mineral phase of bone^[2], and they also lack any significant shrinkage or exotherm upon setting^[2]. GPCs can also be formulated to release clinically beneficial ions such as fluorine^[3], which in dental cements, results in the prevention of secondary caries^[4]. However, previous work into improving the properties of GPCs has resulted in cements with increased mechanical properties^[5] and GPCs which are light cured (Resin Modified GPCs)^[6]. Research has also resulted in increased antibacterial properties by the addition of antimicrobial compounds such as tri-sodium citrate^[7] and chlorhexidine^[8], and by adding antibacterial ions such as zinc (Zn) and silver (Ag) to the glass phase^[7, 9, 10] as there are numerous reports on the toxicity of aluminium in their commercial counterparts^[11, 12]. The objective of this work was to improve the biological response of this Zn-GPC by adding a number of biologically acceptable organic polymers. Organic–inorganic composites have recently attracted attention as bone replacement materials due to their ability to combine several desirable properties,^[13] therefore for this study a number of different natural polymers such as chitin, collagen, cysteine and keratin were considered.

Collagen, a component of the organic phase of bone is known to impart flexibility and toughness to a construct^[14]. Collagen exhibits a number of attractive characteristics which favour it for use as a biomaterial. It has a high tensile strength, high affinity for water, low antigenicity, hemostatic properties, controllable biodegradation, low inflammatory and cytotoxic properties and the ability to promote cellular attachment, growth and differentiation^[15, 16].

Chitin and keratin also provide structural integrity and protection to tissues therefore serving roles analogous to those of collagen in bone. Chitin is a homopolymer^[17] and is one of the most abundant polysaccharides found in nature^[18] occurring as ordered crystalline microfibrils in the exoskeleton of arthropods^[19]. In crustaceans, chitin is found to occur as fibrous material embedded in a six stranded protein helix^[20]. Chitin, together with its deacetylated derivative chitosan, has been shown to be useful as a wound dressing material, drug delivery vehicle and as a candidate for tissue engineering^[21].

Keratin is a high tensile fibrous polymer that maintain cell integrity^[22] and is found in reptiles, birds and mammals, occurring in humans as hair, nail and epidermis (stratum corneum)^[23]. The mechanical stability of keratin and its resistance to biochemical degradation depends on tight packing of the protein chains in α -helix or β -sheet secondary structures and linkage of these structures by disulphide bonds^[24], a covalent bond that forms between two molecules of cysteine. Cysteine is a non-essential amino acid synthesized from methionine^[25]. Cysteine is a precursor of glutathione, an antioxidant primarily located in the cell membrane^[26]. The glutathione-mediated redox cycle is the most important removal system for exogenous and endogenous free radicals^[27]. Cells exposed to acrylic based bone cements produce free radicals^[28] therefore, the incorporation of cysteine into a bone cement may reduce the cement's cytotoxicity.

The main objective of this study is to investigate the effect of the additives chitin, collagen, cysteine and keratin on the material and biological properties of a Ca-Sr-Zn-Si GPC.

2. MATERIALS & METHODS

2.1 Glass Synthesis

A 0.12CaO-0.04SrO-0.36ZnO-0.48SiO₂ glass (BT 101) was formulated by weighing out appropriate amounts of analytical grade reagents (Sigma-Aldrich, Dublin, Ireland) and ball milling (1h). The mixture was then oven dried (100°C, 1h), fired in a platinum crucible (1500°C, 1h) and shock quenched in water. The resulting frit was dried, ground and sieved to retrieve a glass powder with a maximum particle size of 45 μ m.

2.2 Glass Characterisation

2.2.1 *Differential Thermal Analysis (DTA)*

A combined differential thermal analyser-thermal gravimetric analyser (DTA-TGA) (Stanton Redcroft STA 1640, Rheometric Scientific, Epsom, UK) was used to measure the glass transition temperature (T_g) of the glasses. A heating rate of 10°C min⁻¹ was employed using an air atmosphere with alumina in a matched platinum crucible as a reference. Sample measurements were carried out every six seconds between 30°C and 1000°C.

2.2.2 *X-Ray Diffraction (XRD)*

Diffraction patterns were collected using a Philips Xpert MPD Pro 3040/60 X-ray Diffraction Unit (Philips, Netherlands). Disc samples (32mm Ø x 3mm) were prepared by pressing a selected glass powder (<45 μ m) into a backing of ethyl cellulose (8 tonnes, 30s). Samples were then placed on spring-back stainless steel holders with a 10mm mask and were analysed using Cu K α radiation. A generator voltage of 40kV and a tube current of 35mA was employed. Diffractograms were collected in the range 5°<2 θ <80°, at a scan step size of 0.0083° and a step time of 10 s. Any crystalline phases present were identified using JCPDS (Joint Committee for Powder Diffraction Studies) standard diffraction patterns.

2.3 Sample Preparation

2.3.1 Cement Disc Preparation

The cements were prepared by thoroughly mixing the glass powder ($<45\mu\text{m}$) with E9 polyacrylic acid (PAA - M_w , 80,800 $<90\mu\text{m}$, Advanced Healthcare Limited, Kent, UK) and distilled water on a glass plate. The cements were formulated a P:L ratio of 2:1.5 with 50wt% additions of PAA, where 1g of glass powder was mixed with 0.37g E9 PAA and 0.37ml water. The following are the natural polymers used in this study:

- *Chitin (Chi.) - Chitin from crab shells (Sigma-Aldrich, Dublin, Ireland. C7170)*
- *Collagen (Col.) - Bovine achilles tendon, Type V (Sigma-Aldrich, Dublin, Ireland. C4387)*
- *Cysteine (Cys.) - L-Cysteine (Sigma-Aldrich, Dublin, Ireland. C/9150/46)*
- *Keratin (Ker.) - Keratin (MP Biomedicals, Ohio, USA. 902111)*

Each of the polymers was added at 10wt%. Complete mixing was undertaken within 20 seconds. Cement discs were produced by placing the viscous cements in 8 x 2mm split ring moulds, clamping and drying in an oven at (37°C, 1h) prior to use.

Table 1. Cement compositions used in this study.

	<i>Control</i>	<i>Chi.</i>	<i>Col.</i>	<i>Cys.</i>	<i>Ker.</i>
BT 101	1.00	1.00	1.00	1.00	1.00
E9	0.37	0.37	0.37	0.37	0.37
H₂O	0.37	0.37	0.37	0.37	0.37
Additions	0.00	0.075	0.075	0.075	0.075

2.3.2 Preparation of Extracts

Approximately 50g of BT 101 glass was sterilized using γ -irradiation at 25kGray (Isotron ltd, Mayo, Ireland) prior to forming cements. Tissue culture water (Sigma Aldrich, Dublin, Ireland) was selected as the solvent to prepare extracts. The concentration of water was determined using equation 1.

$$V_s = \frac{Sa}{10} \quad \text{Equation 1}$$

Sa = surface area of the cement disc.

Samples ($n=3$) were aseptically immersed in appropriate concentrations of sterile tissue culture water and agitated at ($37^\circ\text{C} \pm 2^\circ\text{C}$) for 1, 7 and 30 days prior to Advanced Surface Area and Porosity (ASAP) analysis and cytotoxicity testing. For ASAP analysis the discs were dried in an oven at $37^\circ\text{C} \pm 2^\circ\text{C}$ and then tested to detect any changes in surface area occurring over time. For cytotoxicity testing 100 μl aliquots ($n=3$) of extract were removed after each time period.

2.4 Working and Setting Times

The setting times (T_s) of the cement series were tested in accordance with ISO9917 which specifies the standard for dental water based cements^[29]. The working time (T_w) of the cements were measured in ambient air using a stopwatch, defined as the period of time from the start of mixing during which it was possible to manipulate the material without having an adverse effect on its properties.

2.5 Mechanical Properties

2.5.1 Compressive Strength

The compressive strengths (σ_c) of the cements were also evaluated in accordance with ISO9917^[29]. Cylindrical Samples were tested after 1, 7 and 30 days. Testing was undertaken on

an Instron 4082 Universal Testing Machine (Instron Ltd., High Wycombe, Bucks, UK) using a 5 kN load cell at a crosshead speed of 1 mm/min⁻¹.

2.5.2 Biaxial Flexural Strength

The flexural strengths (σ_f) of the cements were evaluated by a method described by Williams *et al*^[30]. Cement discs were tested after 1, 7 and 30 days. Testing was undertaken on an Instron 4082 Universal Testing Machine (Instron Ltd., High Wycombe, Bucks, UK) using a 1 kN load cell at a crosshead speed of 1 mm/min⁻¹.

2.6 Surface Area & Solubility Testing

2.6.1 Advanced Surface Area and Porosity (ASAP)

In order to determine the surface area of the cements and any changes occurring over time, the Advanced Surface Area and Porosimetry, ASAP 2010 System analyser (Micrometrics Instrument Corporation, Norcross, USA) was employed. Approximately 60 mg of each set cement disc was analysed and the specific surface area was calculated using the Brunauer-Emmett-Teller (BET) method

2.6.2 Cement Solubility

The cement compositions were mixed ($n=3$) and placed in flexural moulds with approximately 100mm of dental floss was incorporated into each of the GPCs prior to setting. An acetate was then placed above and below the constructs and this was placed between two steel slabs, clamped and put in an oven (37°C, 24h). Once the flexural mould was removed, constructs were weighed. A beaker containing 500ml of water was heated to 100°C. Each construct was then placed in a beaker (100°C, 1h). After the constructs were removed from the beakers, they were dried and re-weighed. Equation 2 was then applied to calculate the solubility of the GPC.

$$\frac{\text{Original Weight} - \text{New Weight}}{(\text{Volume of Disc})} \times \frac{100}{1} \quad \text{Equation 2}$$

2.7 Determination of Bioactivity

2.7.1 Simulated Body Fluid Trial

Simulated body fluid (SBF) was produced in accordance with the procedure outlined by Kokubo *et al*^[31]. The composition of SBF is outlined in table 2. The reagents were dissolved in order, from reagent 1–9, in 500 ml of purified water using a magnetic stirrer. The solution was maintained at 36.5°C. 1 M-HCl was titrated to adjust the pH of the SBF to 7.4. Purified water was then used to adjust the volume of the solution up to 1 L. The SBF was stored in a refrigerator and any that formed precipitates was discarded. Each glass formulation was mixed with E9 PAA at a concentration of 50 wt% to produced samples of cement for immersion in SBF. These cement discs ($n=2$) were produced and were subsequently stored in SBF for 1, 7 and 30 days in an incubator at 37°C. A JOEL JSM-840 Scanning Electron Microscope equipped with a Princeton Gamma Tech (PGT) Energy Dispersive X-ray (EDX) system was used to obtain secondary electron images and carry out chemical analysis of the surface of cement discs. All EDX spectra were collected at 20 kV, using a beam current of 0.26 nA. Quantitative EDX converted the collected spectra into concentration data by using standard reference spectra obtained from pure elements under similar operating parameters.

Table 2. Ionic composition of SBF.

Order	Reagent	Required Amount
1	NaCl	7.996g
2	NaHCO ₃	0.35g
3	KCl	0.224g
4	K ₂ HPO ₄ ·3H ₂ O	0.228g
5	MgCl ₂ ·6H ₂ O	0.305g
6	1M-HCl	40ml
7	CaCl ₂	0.278g
8	Na ₂ SO ₄	0.071g
9	NH ₂ C(CH ₂ OH) ₃	6.057g

2.7.2 *In vitro* assessment of Extracts

The established cell line L-929 (American Type Culture collection CCL 1 fibroblast, NCTC clone 929) was used in this study as required by ISO10993 part 5 ^[32]. Cells were maintained on a regular feeding regime in a cell culture incubator at 37°C/5% CO₂/95% air atmosphere. The culture media used was M199 (Sigma Aldrich, Ireland) supplemented with 10% fetal bovine serum (Sigma Aldrich, Ireland) and 1% (2 mM) L-glutamine (Sigma Aldrich, Ireland). The cytotoxicity of cement extracts was evaluated using the Methyl Tetrazolium (MTT) assay in 24 well plates. Undiluted extract (100µl aliquots) were added into wells containing L929 cells in culture medium in triplicate. The prepared plates were incubated for 24h at 37°C/5% CO₂. The MTT assay was added in an amount equal to 10% of the culture medium volume/well. The cultures were then re-incubated for a further 2h (37°C/5% CO₂). Next, the cultures were removed from the incubator and the resultant formazan crystals were dissolved by adding an amount of MTT Solubilization Solution (10% Triton x-100 in Acidic Isopropanol. (0.1 n HCl)) equal to the original culture medium volume. Once the crystals were fully dissolved, the absorbance was measured at a wavelength of 570 nm. Controls consisted of 100µl aliquots of tissue culture water, and cells were assumed to have metabolic activities of 100%.

2.8 Statistical Analysis

One-way analysis of variance (ANOVA) was employed to compare the properties of the modified cements to the control BT 101 cement, and in some cases to any changes occurring with respect to maturation. Comparison of relevant means was performed using the post hoc Bonferroni test. Differences between groups was deemed significant when $p \leq 0.05$. Statistical analysis was performed using SPSS software for windows version 16 (SPSS Inc. Chicago, IL).

3. RESULTS & DISCUSSION

The objective of this work was to investigate any changes in relation to handling, mechanical and biological properties, as a result of the addition of organic polymers to a Zn-GPC. The glass was characterized using Differential Thermal Analysis (DTA) and X-ray Diffraction (XRD) both before and after γ -sterilization in order to determine any immediate structural changes. Figure 1 shows the characterization of the glass phase. Characterization revealed that little change occurred in the glass as a result of γ -sterilization. XRD determined that the glass was initially amorphous and that no changes occurred as a result of sterilization. DTA revealed a slight drop in T_g from 662-650°C, however this is likely an insignificant difference. Previous work on similar glass compositions resulted in little change when exposed to slightly higher doses of γ -irradiation^[33].

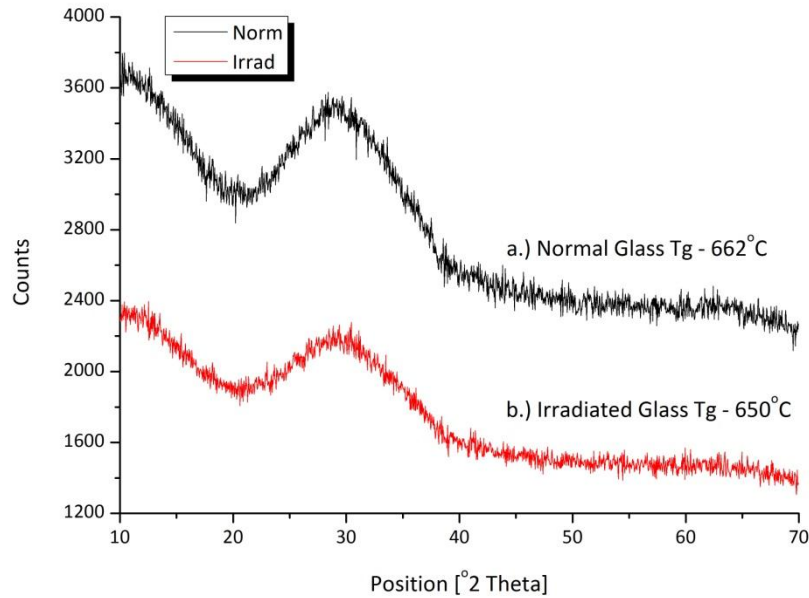


Figure 1. Characterization of glass used for cement series.

The handling properties, the working time (T_w) and the setting time (T_s) are important characteristics in cementation. Extended T_s can result in increased exposure time resulting in septic complications^[34], while T_s that are too short can mechanically compromise the material. Figure 2 illustrates the T_w and T_s of the control cement (BT 101) and cements containing

additions of the organic polymers. The T_w of BT 101 was found to be 36s. The cements denoted *Chi.*, *Col.*, *Cys.*, and *Ker.* were statistically compared to BT 101 and it was found that significance was only reached with *Chi.* where the T_w was found to be 30s ($p \leq 0.016$). The remaining cements, *Col.*, *Cys.*, and *Ker.*, achieved T_w of 32s, 38s and 33s respectively.

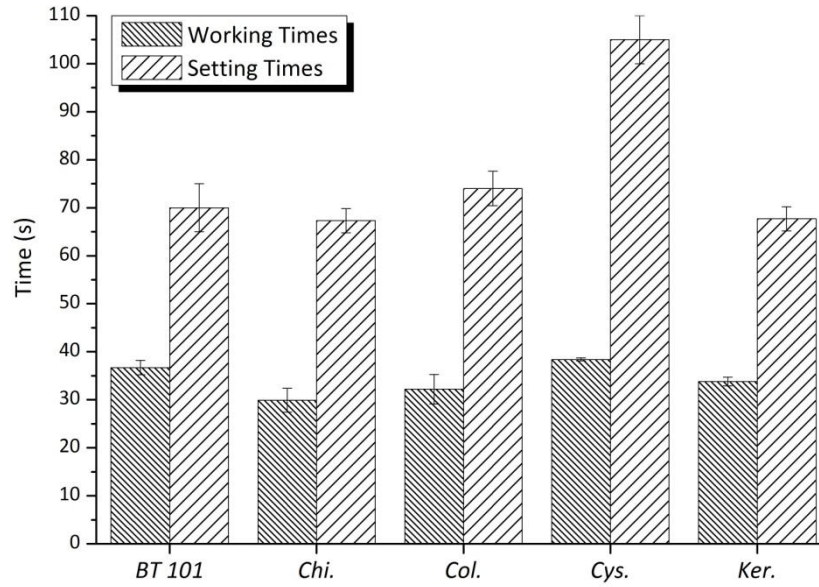


Figure 2. Working and setting times of cement series.

In relation to the T_s , there was a significant difference with only one of the modified cements when compared to the T_s of BT 101 (70s). *Cys.* attained a T_s of 105s ($p \leq 0.0001$). The remaining cements *Chi.*, *Col.*, and *Ker.* had a T_s of 67s, 74s and 67s respectively. The T_w and T_s determined here are considerably shorter than some of the commercially available GPCs. This is due to factors relating to the composition of the glass and the presence of additives in the commercial materials such as tartaric acid^[35].

Mechanical testing was then performed considering compression (σ_c) and biaxial flexural (σ_f) testing. Firstly comparisons were made to determine if any significant change in σ_c occurred with respect to maturation. Figure 3a shows the σ_c results.

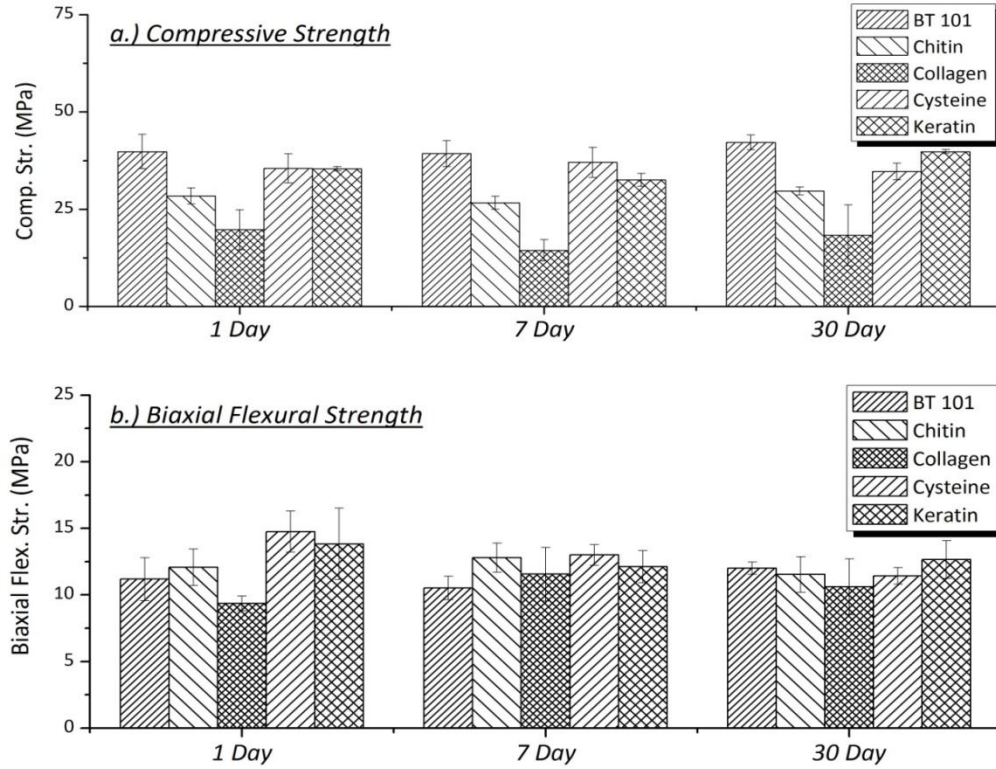


Figure 3. a.) Compressive strength and b.) Biaxial flexural strength of modified cement series.

For the control cement (BT 101) a maximum σ_c of 42 MPa was attained after 30 days, however there was found to be no significant change in strength over 1, 7 and 30 days. For *Chi.* a maximum strength of 29 MPa was found after 30 days and only reached significance between 7 and 30 days ($p \leq 0.036$). Both *Col.* and *Cys.* achieved maximum strengths of 19 MPa (1 day) and 37 MPa (7 day) respectively, however there was no significant change in strength over time. *Ker.* was found to be significantly different at each time frame with strengths for 1 day at 35 MPa reduced to 32 MPa after 7 days ($p \leq 0.007$), and then recovering to 39 MPa after 30 days ($p \leq 0.0001$). In comparing each of the modified cements to the control (BT 101) at 1 day, it was found that the strength of *Chi.* ($p \leq 0.005$) and *Col.* ($p \leq 0.0001$) reduced significantly. At 7 days *Chi.* ($p \leq 0.0001$), *Col.* ($p \leq 0.0001$) and *Ker.* ($p \leq 0.017$) were found to decrease in strength. At 30 days a similar trend occurred where *Chi.* ($p \leq 0.001$) and *Col.* ($p \leq 0.0001$) reduced in strength significantly. From σ_c it was found that there was no significant increase in strength either with respect to maturation or between cements when compared to the control, however, cements were found to significantly reduce in strength particularly with additions of *Chi.* and *Col.*. In the case

of *Chi.* the reduction in strength may be due to its ability to chelate $\text{Ca}^{[36]}$, which in these cements is important as it forms ionic crosslinks which give rise to mechanical strength. In the case of *Col.*, it's presence within the cement structure, and it's insolubility at body temperature^[37], may contribute to reduced crosslinking within the cement matrix resulting in reduced strength.

Biaxial flexural testing (figure 3b) revealed that there was little change in σ_f with respect to maturation for each cement. There was no change in BT 101, *Chi.*, *Col.*, and *Ker.* over time. The only significant change occurred when comparing *Cys.* at 1 and 30 day where the cement reduced in strength from 14-11 MPa ($p \leq 0.001$). In comparing each of the modified cements to BT 101 at each time frame it was found that at 1 day, *Cys.* (14 MPa) showed a significant increase ($p \leq 0.042$) compared to BT 101 (11 MPa). At 7 and 30 days there was no significant difference between BT 101 and any of the modified cements. It is also evident that changes in σ_c occurred while little changes are evident in σ_f . This may be attributed to the test method. The σ_f is cited in the literature as being a more discriminatory test, as compressive samples tend to fail in both shear and tension^[38, 39].

Testing was then performed to determine any changes that might have occurred in the cement structure as a result of the addition of each of the organic polymers. For this, solubility testing and surface area analysis were undertaken as both of these factors will have an influence on the mechanical properties and in particular the bioactivity. Figure 4a shows any changes in the cement's surface area with respect to maturation, figure 4b shows the solubility of each of the cements.

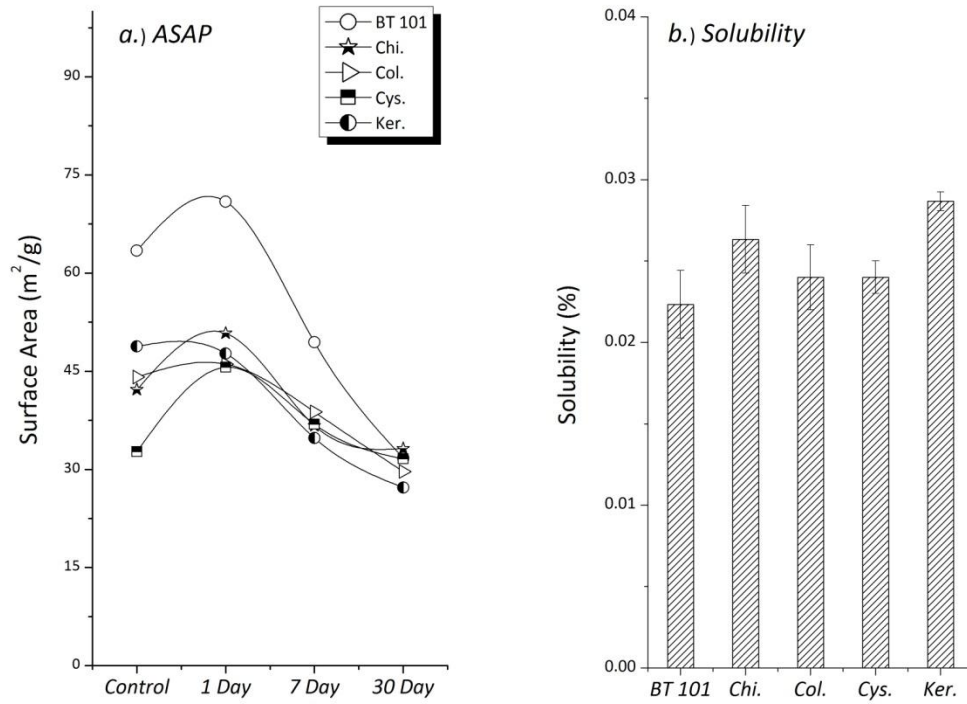


Figure 4. Surface area and solubility of cement series as determined by ASAP.

From figure 4a it can be seen that a similar trend occurs in the majority of the cements where there is an initial increase in the surface area of the cement after 1 day exposure in water, however this decreases at 7 and 30 days. The control cement BT 101 was found to have the highest surface area ($70 \text{ m}^2/\text{g}$) at 1 day, and also to be less soluble (0.022%) than each of the modified cements. The higher surface area of BT 101 is likely due to the higher concentration of micron sized glass particles in the cement compared to the modified cements. The soluble glass particles in these cements degrade over time to release cations into the surrounding aqueous environment. After 7 ($49 \text{ m}^2/\text{g}$) and 14 days ($31 \text{ m}^2/\text{g}$) the glass particles are dissolved resulting in the metal cations being suspended in solution thereby reducing the surface area within the cement. This is the likely reason for the reduced surface area with respect to time, and a similar trend is found in relation to each of the modified cements. It is likely that the reduced surface areas of *Chi.* ($50 \text{ m}^2/\text{g}$), *Col.* ($46 \text{ m}^2/\text{g}$), *Cys.* ($45 \text{ m}^2/\text{g}$) and *Ker.* ($47 \text{ m}^2/\text{g}$) at 1 day is due to larger internal porosity as the additives are large molecules and in addition their place in the cement displaces the balance of glass particles that would be present in the control cement. There was also found to be a slight increase in the solubility of the cements with the addition of *Chi.*

(0.026%) and *Ker.*, however *Ker.* at 0.029% ($p \leq 0.009$), was the only modified cement to reach significance when compared to the control.

The first step in determining the bioactivity of the cements was to test for surface apatite precipitation when immersed in Simulated Body Fluid (SBF). SBF is a solution designed by Kokubo *et al*, which has an ionic composition similar to that of blood plasma^[31]. Calcium phosphate (CaP) surface deposition on glasses and glass-ceramics is seen as a pre-requisite to bone bonding, however a number of conditions must be present, such as pH (4.2-12), Ca^{2+} and PO_4^{2-} above the solubility limit for hydroxyapatite (HA) and a negatively charged surface^[31, 40, 41]. Figure 5 shows the SEM and corresponding quantitative EDX (Norm wt%) of BT 101. *Chi.*, *Col.*, *Cys.* and *Ker.* after 1 and 30 days.

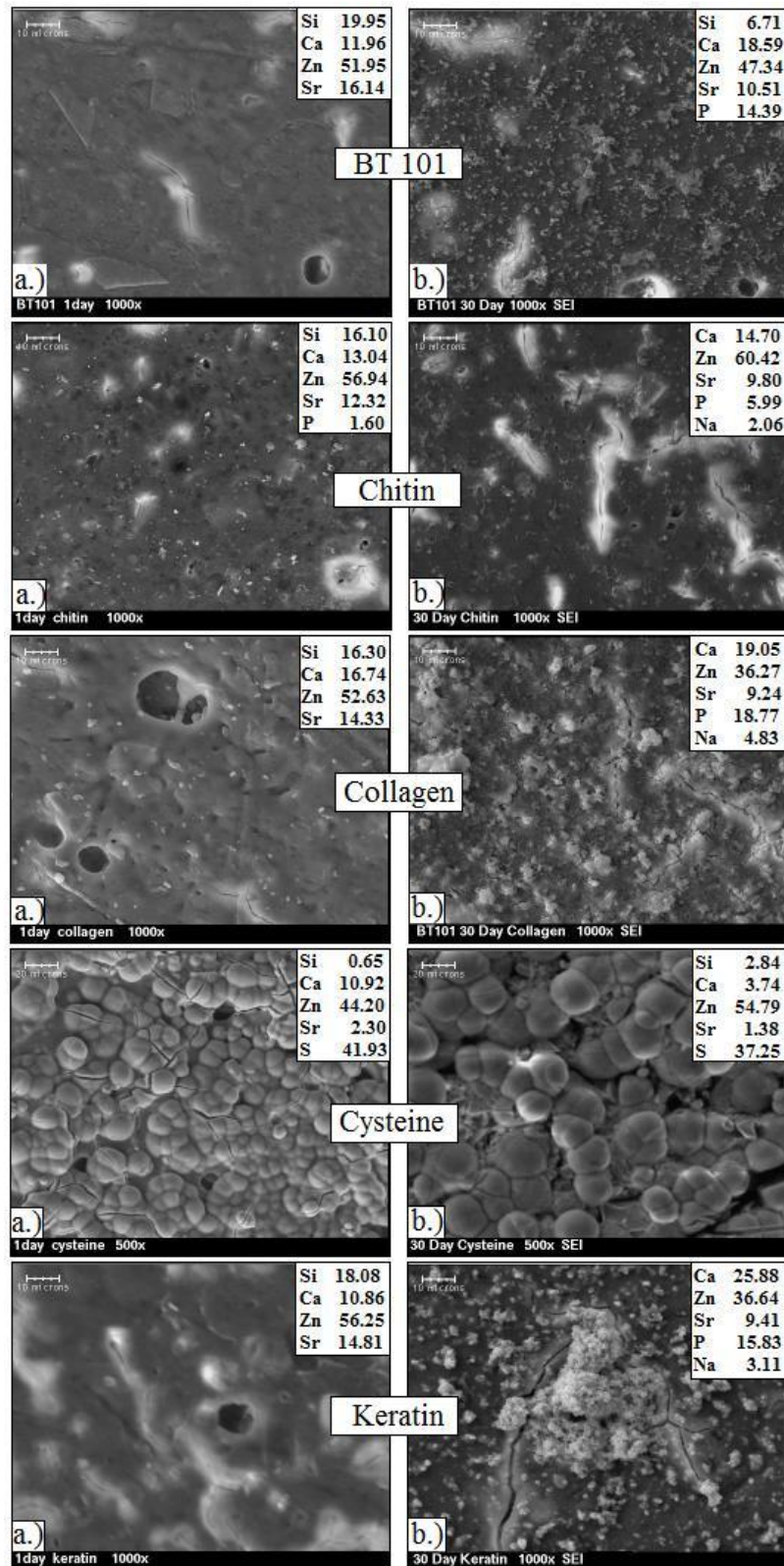


Figure 5. SBF results of modified cement series after a.) 1day and b.) 30 days.

From figure 5 it can be seen that BT 101 showed no CaP surface layer deposition after 1 day, however CaP did form after 7 days and increased up to 30 days (14.39 wt%). Considering BT 101, it is likely that surface deposition occurs due to the presence of Ca in the glass phase and soluble silica which forms Si-OH groups on the surface of the material^[42, 43]. Ca release from the cements increases the Ca^{2+} concentration in the SBF and upon reaching the saturation limit, precipitation occurs and the negatively charged surface binds Ca^{2+} and PO_4^{2-} . In relation to these cements, it has previously been reported that Zn from the cement inhibits the crystallization of this surface layer^[44].

Chi. exhibited CaP surface layer at each time frame, 1, 7 and 30 days (5.99 wt%). Furlan *et al* cites that *Chi.* has the ability to chelate metal cations (Mg^{2+} , Ca^{2+} , Zn^{2+} and Al^{3+}), suggesting that it could be used for waste water treatment, while their own research demonstrates the superior ability of PAA and *Chi.* to chelate Ca. The high binding affinity is due to the action of plural functional groups, carboxylic and acetamido groups^[36]. *Col.* did not produce a surface layer after 1 day, however after 7 days this layer was present and after 30 days (18.77 wt%) the surface was covered. *Col.* is the principal component of the organic phase of bone and has been considered in numerous studies to improve different properties of bone cements^[37, 45-47]. However, *Col.* is composed mainly of hydrophobic glycine and proline residues, which have limited capacity for interaction with Ca^{37} . While the addition of *Col.* may not have added to surface precipitation at 1 day, its inability to bind Ca may have contributed to the increased precipitation of CaP after 30 days as a result of a higher concentration of Ca suspended in solution. However, a study has been conducted where the use of collagen has influenced the precipitation of HA. Using Type I Collagen as a coating on titanium metallic implants, an increase in the rate of bone remodeling was observed, which was attributed to its excellent biocompatibility and osteoconductivity^[48].

Cys. did not produce a CaP layer at any time point, however after only 1 day the surface layer was covered in calcium/zinc sulfate, and this remained relatively stable up to 30 days. *Ker.* performed similarly to BT 101 where there was no surface deposition after 1 day however after 30 days there was a high concentration of CaP (15.83 wt%). It is likely that *Ker.* performed in a similar fashion to *Col.*, where it did not participate in the precipitation of CaP and this is attributed to the components of the cement.

Cell culture studies were performed on cement extracts after 1, 7 and 30 days as cement discs alone proved to reduce cell viability to almost zero. This is due to the high ion release rate from these cements causing toxicity within the enclosed wells. Figure 6 shows the results of the cytotoxicity testing on the control and modified cements.

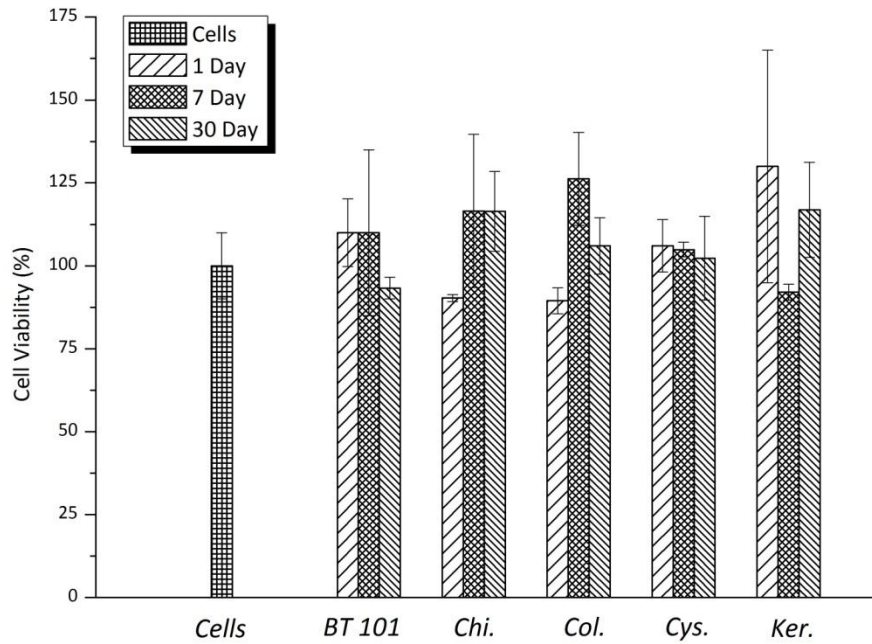


Figure 6. Cytotoxicity testing of cements series determined by MTT assay.

From figure 6 it can be seen that there was little change in the cell viability of the cements. There was no significant change in cell viability for BT 101, *Chi.*, *Cys.*, and *Ker.* *Col.* was the only cement to reach significance when compared to BT 101 ($p \leq 0.0001$) at 7 days. The relatively small changes occurring in cell viability may be due to the insolubility of some of the additives, *Col.* in particular has been described as being particularly insoluble at physiological pH^[37]. *Chi.* exhibited no significant change compared to the control, however a significant increase was observed between 1-7 days ($p \leq 0.032$) and 1-30 days ($p \leq 0.033$). However insolubility is also characteristic of *Chi.* in an aqueous environment^[49]. *Cys.* showed no significant changes either over time or compared to BT 101. N-acetyl *Cys.* has previously been added to bone cements and was found reduce the cytotoxicity and improve the osteoconductivity without affecting the mechanical properties. However this occurs in PMMA based cements as the sulfhydryl groups

from *Cys.* can inactivate the monomer components^[50]. *Cys.* can also eliminate free radicals by enabling the self detoxification of bone cement by preventing free radicals from being released from the material or by enhancing cellular antioxidant defense^[50, 51]. Although *Ker.* achieved the highest cell viability (130%), this increase was found to be insignificant. *Ker.* has previously been used to produce biodegradable scaffolds for biomaterial and as cell cultivation scaffolds as it is a structural protein similar to collagen^[52-54]. However in this case it is likely that *Ker.* remains entrapped within the cement matrix. An evident observation that can be made from these results is that little change occurs in the cements with respect to maturation, which suggests that ions released from these cements reach a stable level after 1 day and do not enhance toxicity after 7 and 30 days.

To conclude, the addition of *Chi.*, *Col.*, *Cys.*, and *Ker.* resulted in little change to the T_w and T_s , however the compressive strength was found to decrease slightly. No significant change was observed in the flexural test, however ASAP and solubility tests revealed that the cements may undergo some structural changes as a result of these additions. Bioactivity was increased with the addition of *Chi.* and *Col.* in SBF, however only *Col.* produced significant changes in cell culture. In future perhaps the addition of organic polymers suspended in the liquid phase as part of the cement may yield improved results in this type of study.

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